Sampling procedures for the diagnosis of banana Xanthomonas wilt

technical bulletin

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Cover pages: banana wilt symptoms caused by *Xanthomonas campestris* pv. *musacearum*

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Sampling procedures for the diagnosis of banana xanthomonas wilt

TECHNICAL BULLETIN

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Contents-

1. Introduction to BXW Surveys

Bacterial Xanthomonas wilt (BXW) disease is caused by *Xanthomonas campestri*s pv. *musacearum t*hat causes loss both through death of the plant and rotting of edible/marketable banana fruit. Symptom development under favourable conditions can be observed within 2-5 weeks under field conditions and 2-3 weeks under glasshouse conditions. BXW is currently restricted to Africa, and it was previously known only to attack Ensete (*Ensete ventricosum*) and bananas in Ethiopia. In a period of four years since its discovery in central Uganda in 2001, the disease quickly spread throughout the Eastern, Central and North-Western districts of the country and neighbouring countries. The disease was first reported in Ethiopia in 1968 (Yirgou and Bradbury, 1968), in 2001 in Uganda, 2004 in DR Congo, 2005 in Rwanda, 2006 in Tanzania and Kenya and 2009 in Burundi. Outbreaks of the disease are periodically reported.

Wilt symptoms in bananas can be caused by different pathogens. Therefore, a correct diagnosis is essential for recommendation and/or application of correct and timely management practices against the disease. The present bulletin describes procedures for the visual survey to aid in the collection of samples from banana plants with BXW symptoms that are sent to a central diagnostic laboratory for molecular-based confirmatory tests. Information is also given on the common methods of isolation of the pathogen from plant, insects and soil materials.

We believe that the information given in this technical publication will be useful for extension services, analysts at plant disease diagnostic laboratories and certification agencies and for research workers, teachers and students of plant pathology in the sampling methodologies for this important pathogen of banana.

The procedures here presented were developed under a cooperative research project (Enreca LIFE-731) for the enhancement of research capacity in the diagnosis and management of bacterial diseases in Eastern Africa with partners from: (i) Danish Seed Health Centre, Faculty of Science, University of Copenhagen, Denmark, (ii) Eduardo Mondlane University, Mozambique, (iii) Sokoine University of Agriculture, Tanzania and (iv) National Research Laboratories, Kawanda Research Institute, Uganda.

Common Names of the Disease

Banana *Xanthomonas* wilt (BXW), banana bacterial wilt (BBW), enset wilt, *Xanthomonas* wilt of enset and banana.

Local names used by farmers: Kiwotoka (Uganda); Murcha bacteriana da bananeira (Mozambique); Mnyauko wa migomba unaosbabisishwa na bakteria (Tanzania)

Pathogen

Xanthomonas campestris pv. *musacearum*; Pathotype strain: *X. campestris* pv. *musacearum* NCPPB 2005

Proposed name: *Xanthomonas vasicola* pv. *musacearum*

Host Plants

Natural hosts: cultivated ensete (*Ensete ventricosum*) and banana (*Musa* spp.); ginger (*Zingiber officinale*), ornamental species by artificial inoculation (e.g., *Canna orchoides*).

Quarantine Status

X. campestris pv. *musacearum* has not been included in the A1 or A2 lists of recommended quarantine pests of the IAPSC (InterAfrican Phytosanitary Council). Within the pest risk area (PRA), Zanzibar alone has placed restrictions on the import of banana to reduce the risk of entry of *Xanthomonas* wilt. The pathogen has not been listed as a notifiable pathogen by countries within the PRA area. No pest free areas are designated. The use of terminology such as endemic and outbreak is in common usage.

France proposed *X. campestris* pv. *musacearum* for inclusion in the European Union list of recommended quarantine pests, with respect to France's overseas departments and regions of La Reunion, Guadeloupe, Martinique and French Guiana. This status is now under consideration by a working group of the European Food Safety Authority (EFSA) (European and Mediterranean Plant Protection Organisation).

PRA area: The PRA area covers the following Eastern, Central and Southern African countries Angola, Botswana, Burundi, Democratic Republic of Congo, Eritrea, Ethiopia, Kenya, Madagascar, Malawi, Mozambique, Namibia, Rwanda, Somalia, Sudan, Swaziland, Republic of South Africa, Tanzania, Uganda, Zambia and Zimbabwe.

2. Visual Surveys for BXW

For a preliminary indication of plant infection look for yellow bacterial ooze from cross sections of pseudostems, fruit fingers, bracts from inflorescence, petioles and signs of wilt on leaves. Collect plant parts (see symptoms description) (Fig. 1 & Fig. 2).

Symptoms of BXW

On leaves: Symptoms are characterised by a dull yellow wilt of the leaves, and may appear as burnt; often the central upper leaf may wilt first and followed by yellowing and wilting of newly expanded leaves of the infected plant. BXW wilt-infected leaves may bend about one-third the leaf length from the leaf tip (Fig. 1a & 1b). Similar symptoms are observed on ensete the original host (Fig. 2).

On flowers: The bracts of infected male buds may appear discoloured, deep purple to dark brown or grey, with drying rot. Flowers shrivel and eventually die (1c top right). Male bud infection proceeds through the rachis which appears discoloured and spread to the pseudostem down to the corm from where it moves into suckers adjoined to the mother plant.

On fruits: Premature and uneven ripening of fruit is observed (Fig. 1a top left and 1c); fruits show brown and/or yellow-orange discolouration with bacterial ooze (pus-like); fruits rotten and harden and are not suitable for human or animal consumption.

On pseudostems: Internal symptoms in cut pseudostems are a yellow-orange discoloration of vascular tissue (vascular streaking) with some bacterial ooze (Fig. 1d & 1e). Massive yellow-orange bacterial ooze in severely affected plants can be released after 5-30 minutes.

Figure 1a: Affected BXW banana plants surveyed in Uganda during 2009-2010; banana wilt symptoms on leaves and early maturation of banana fingers (Top left); affected leaves appear as fire burnt; bent or snap yellow leaves at the middle of the blade (Photographs John Adriko)

Figure 1b: Severe wilt symptoms in bananas observed during field surveys of Northern Tanzania, 2010 (Photographs, Ernest Mbega)

Figure 1c: Early maturity of fingers and vascular discolouration of fruits by BXW pathogen; observe inflorescence with severely affected flowers and fingers (Photographs, Ernest Mbega and John Adriko)

Figure 1d: Vascular symptoms in bananas caused by the BXW pathogen *Xanthomo campestris* pv. *musacearum*. Observe, discoloured infected pseudostem region, in cross and longitudinal sections (Photographs, Ernest Mbega)

Figure 1e: Vascular symptoms in bananas caused by the BXW pathogen. Observe, the large amount of bacterial ooze from cross sections of pseudostems, detached bracts and rachis of inflorescence (below) affected by the pathogen *Xanthomonas campestris* pv. *musacearum* (Photographs, John Adriko: top left; J. Kubiriba top right; below, Ernest Mbega)

Figure 2: Ensete plants with BXW symptoms, Ethiopia (Courtesy: Kidish Bobosha, Ethiopia)

3. Other Wilt Diseases

Bacterial Diseases

Moko disease or bacterial wilt: the disease is caused by *Ralstonia solanacearum* and it is characterised by pale yellow brown discoloration of young leaves in actively growing plants, and tissue collapse within a week. Young suckers may be blackened stunted or twisted. Fruits are brown and show black dry rot on cross sections. Mature plants pseudostems show white bacterial ooze (Fig. 3)

Bacterial blood disease: caused by *Xanthomonas campestris* pv. *celebensis* has been reported in Indonesia; this disease is similar to moko disease but a reddish colour bacterial exudate from fruits is observed.

Fungal Diseases

Fusarium wilt: Wilt symptoms caused by *F. oxysporum* f.sp. *cubense* frequently observed in Uganda and is prevalent in many other parts of Africa. In Uganda, Fusarium wilt is prevalent on introduced banana cultivars that are used primarily as dessert bananas and for brewing, e.g. Kayinja (Fig. 4). *Fusarium oxysporum* f.sp. *cubense* - mediated wilting starts from older leaves and spreads to younger leaves in contrast to BXW infections that can appear on younger leaves; infected plants leaves bend at the petioles while for BXW leaves bend at the middle of the blade.

'Matoke wilt': also called as 'the disorder' reported in 1993 of symptoms somewhat similar to those of Fusarium wilt were observed on the indigenous and dominant highland cooking bananas (AAA-EA) in the Western Uganda highlands. It can affect non-indigenous as well as indigenous banana types. *Fusarium pallidoroseum* has frequently been isolated from these tissues, but it has not been found to be pathogenic in host inoculations.

False Panama disorder: can easily be confused with Fusarium wilt and in most cases yellowing of leaves starts with the lower or older leaves. The margin of each leaf turns pale green to yellow, necrotic strips surrounded by a yellow margin occur and eventually the leaf dies off. The lower leaves die completely and hang down the pseudostem as a skirt. Wine-red discolouration of cross –sectioned pseudostems without gum in pockets have been observed. Up to now it has not been possible to isolate any pathogens from affected plants. It has been suggested that a combination of stress factors such as drought, water-logging, soil compaction, nutritional imbalance in combination with low temperatures, could be the cause of the disorder.

Figure 3: Bacterial ooze of whitish colour on cross sectioned pseudostem of banana plant affected by *Ralstonia solanacearum*, the cause of bacterial wilt and moko disease of banana (Courtesy of Le Huong, Vietnam)

Figure 4: Banana plants affected by *Fusarium wilt* in Uganda (Photographs: John Adriko and Jerome Kubiriba): observe wilt symptoms that start in older leaves which bend at the petiole, and dark discoloured vascular bundles in cross section of affected pseudostems of infected banana plants

4. Collection of Samples

Timing and Selection of Plant Parts

The disease is readily detected in pseudostems and petioles all year round. The pathogen is of a systemic nature. This may involve the upward movement of bacteria through the vascular tissues if infection occurs in the lower parts of the plants (rhizome or pseudostem) or the downward movement of bacteria if infection occurs through the inflorescence.

Sampling areas

The areas sampled for the presence of BXW (Fig. 6) in Uganda districts, Tanzania and Mozambique in the period 2009-2010. The pathogen was detected in the sampled areas of Uganda and Tanzania, but not in Mozambique at the time of the visits.

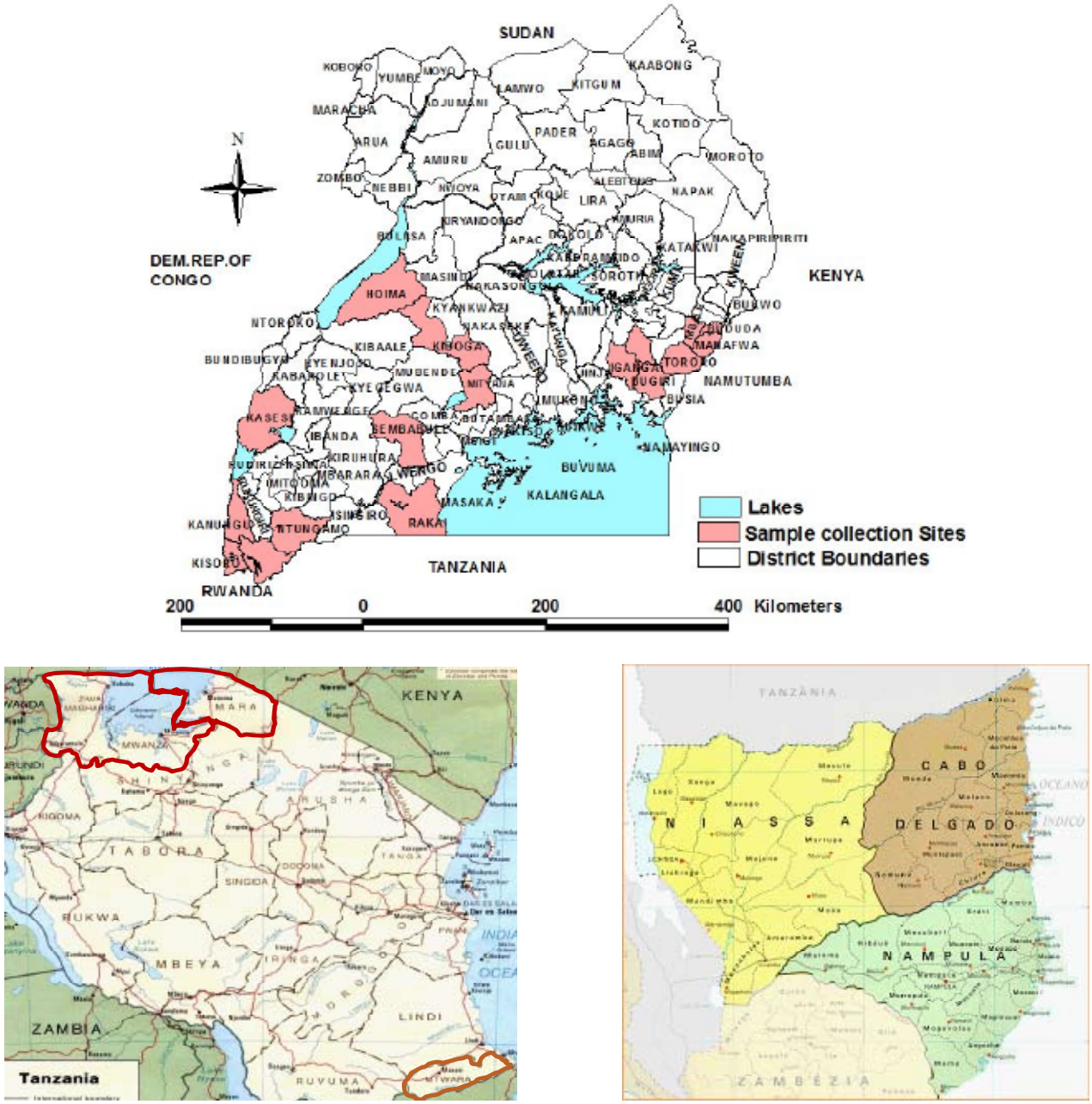

Figure 5: Areas of surveys conducted in 2009-2010 in regions for banana plants in Southern districts of Uganda (Top); Northern and Southern Tanzania (Mwanza, Mara; Mtwara & Ruvuma), (bottom left) and Northern provinces of Mozambique (Niassa, Cabo Delgado & Nampula), bottom right

Sampling with silica gel

1. Label and identify each tube sample with the date of collection, name of the variety, locality and name of extension officer or agency
2. Select several (8-10) symptomatic or asymptomatic leaves, petioles, affected pseudostems, inflorescence, peduncles, bracts of male flowers, petioles, leaves or fingers; root samples should be washed and excess of water removed prior packing
3. Cut the selected tissue from different selected plant parts with clean and disinfected tools
4. Each piece of tissue should be approximately 5 cm long
5. Place individual samples into a clean labelled, test tube with silica crystals and with cotton layer to keep the sample dry after collection (Fig. 6)
6. If a relatively small number of samples are to be assayed, individual plant samples would offer the highest degree of sensitivity in detecting BXW
7. Alternatively combine plant parts from the individual plants each of five parts into one composite sample
8. Ship the sampled material as soon as possible to the near diagnostic laboratory.

Note: suitable for isolation of the pathogen, identification conducted with genus *Xanthomonas* or species specific primers for *X.c.* pv. *musacearum (*Adriko et al., 2011, Adriko et al., 2012; Ernest et al., 2012)

Sampling without silica gel

1. Select plant parts and keep samples as cool as possible to prevent drying and microbial degradation. Portions of the affected parts, including the root system are collected in plastic bags and wrapped with paper and kept cool in order to avoid tissue decomposition.
2.. For shipment of plant parts keep samples cooled with ice packs, taking care to cover the ice packs with newspaper to prevent contact freezing.

Sampling from soil

1. A sample should consist of minimum of 50-100g of soil collected from the rhizome and/or rhizosphere area (prepared from composited samples)
2. Samples are collected in plastic bags and kept as cool as possible as indicated under sampling without silica gel

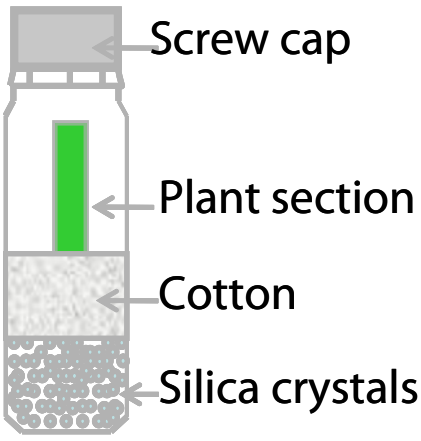

Figure 6: Diagram of sampling device for the collection of BXW affected banana plant material. Collection of field samples for the isolation of *Xanthomonas campestris* pv. *musacearum*

5. Isolation of the Pathogen

From Plants

Isolation of the pathogen *X. campestris* pv. *musacearum* has been conducted onto agar substrates of general composition (e.g., NA, SPA, YDC). However, its isolation can often be difficult from severely affected plant parts and even on semi-selective agar substrates such as CCA (Fig. 7). These substrates have improved the isolation of the BXW (Recipes in Appendix). The pathogen has been isolated from the pseudostem nectar and from the ooze exuding through the cushions revealed by the fallen male flowers and the fresh openings made by the fallen bracts of both the male and female flowers. However, in the present surveys isolation was easier to obtain from affected leaf blade tissue.

1. Select areas for isolation: use affected plant parts and select areas where the symptoms are not so advanced and are not dominated by secondary microbes (saprophytes)
2. Remove excised plant material (2-5mm^2) sections aseptically from the internal region of an infected area (stem in cross or longitudinal-section) and placed in a test tube with sterile water for at least 15 min
3. Take loopfuls (50-100µl) samples are taken for isolations made onto nutrient agar plates. Use a semi-selective agar substrate if high levels of saprophytes are expected
4. Incubate the inoculated agar plates at 27-30°C for 48 hours and observe for the presence of individual colonies
5. Transfer yellow pigmented colonies for purification to YDC agar plates for 24-48 hours
6. Maintain cultures in sealed test tubes refrigerated (5-7°C) or keep in Protect™ bacterial preservers until use.

From Soil and Plant Debris

X. campestris pv. *musacearum* populations decline rapidly in non-sterile soil, persisting for only 35 days; from plant debris, the bacterium appears to survive for only 21 days when buried or on the soil surface.

1. Collect soil samples, allow to settle and serially dilute in water (e.g., 3-4 x 1:10)
2. Streak loopuls of the samples onto a semi-selective agar substrate as previously described.

From Insects

Insects visiting banana flowers have been identified as potential vectors in Uganda. The most common insects were stingless bees (Family *Apidae*), fruit flies (Family *Drosophilidae*) and grass flies (Family *Chloropidae*). The bacterium can be isolated from stingless bees, honeybees (*Apis mellifera*), fruit flies and grass flies that had been collected from male flowers of symptomatic and asymptomatic plants:

1. Crush insects collected in test tubes as for plant parts (alternatively silica crystals are substituted with calcium chloride)
2. Suspend in sterile water (e.g., 3-4 x 1:10) and streak loopfuls of the suspension or spread with the help of a glass rod 50-100µl samples onto selected agar substrate(s) but preferably onto CCA.
3. Incubate plates at 27-30°C for 48 hours or longer and observe for the presence of individual colonies. Transfer colonies for purification to YDC agar plate and submit cultures for a complete identification of the pathogen.

Figure 7 : Growth of *Xanthomonas campestris* pv. *musacearum* isolated from bananas from Uganda: on nutrient agar (left), on semi-selective CCA medium (centre), and YDC agar (right); observe the yellow, mucoid, dome-shaped and circular colony growth

6. Recipes of Agar Substrates

Nutrient agar (NA):

Yeast extract.....3 g
Peptone ..... 5 g
NaCl.....5 g
Agar..... 5 g
Distilled water..... 1 L

Adjust pH to 7.4 and sterilise by autoclaving at 121 °C for 15 min

Yeast dextrose calcium carbonate (YDC) agar:

1. Yeast extract..... 10 g
$CaCO_3$ light powder..... 20 g
Agar ..... 20 g
Distilled water.....950 mL

2. Dextrose (L-glucose) ..... 20 g
Distilled water ..... 50 mL

Autoclave the two solutions separately and mix well when the temperature of the medium is 40-50 °C

Sucrose peptone agar (SPA):

Sucrose..... 20 g
Peptone.....5 g
K_2HPO_4 (anhydrous)..... 0.5 g
$MgSO_4 . 7H_2O$..... 0.25 g
Agar..... 12 g
Distilled water..... 1 L

A*djust pH 7.2-7-4 and sterilise by autoclaving at 121 °C for 15 min*

Cellobiose-cepalexin agar (CCA)

Yeast extract.....1 g
Glucose..... 1 g
Peptone.....1 g
NH_4Cl .....1 g
$MgSO_4 . 7H_2O$.....1 g
K_2HPO_4 (anhydrous)..... 0.5 g
Cellobiose ..... 10 g
Agar .....14 g
Distilled water.....950 mL

2 . Distilled water .....50 ml
Cephalexin.....40 mg
5-fluorouracil .....10 mg
Cycloheximide.....120 mg

Autoclave the two solutions separately and mix well when the temperature of the medium is 40-50 °C

7. Shipment of Samples to Diagnostic Laboratory

Shipment of Plant Samples

1. Ship the collected samples or cultures for delivery within 1-2 days to the near diagnostic laboratory.
2. Include a cover letter with your contact information.

Submitting Samples for PCR Assays

1. Molecular level tests confirm the presence of the disease in the sample collected
2. For further diagnosis and best results of PCR assays (Adriko et al., 2011 & Adriko et al., 2012), please submit a representative sample of the infected material* to be tested as previously described under silica gel and test tubes collection of samples (Fig. 6) which can be provided by the near extension service officer in the region and after agreement with a central diagnostic laboratory.
3. To avoid any delays, submit enough plant material to complete the assay.

   **Alternatively those with facilities for isolation of the pathogen in pure culture can send the sample in an agar slant or stubs/petri dish properly sealed and labelled.*

Time for Completion of Assays

1. The assay with BXW species specific primers may take 1-2 days without pathogen isolation
2. Pathogen isolation may require 6-7 days plus 1-2 days of PCR assay: total 7-9 days
3. Pathogenicity test with host plants may take 21-45 days.

Submitting Samples for Other Tests

1. If fungal pathogens are suspected, a fungal evaluation and subsequent assays can be performed. Please submit a sample consisting of, portions of the affected parts, including the root system collected in plastic bags and wrapped with paper and kept cool in order to avoid tissue decomposition. A fungal test may take approximately one week.
2. For our service brochures or additional information on testing costs, please contact us by e-mail or call us to our delivery address above mentioned.

Company name: _____ Your name:_____

Address:_____ Telephone #: _____

Fax #: _____E-mail: _____ Variety: _____

Species:_____ Plot location:_____

Delivery Address:

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